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Alleviating tumor hypoxia with perfluorocarbon-based oxygen carriers

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Abstract

Hypoxia is a major impediment to many foremost cancer treatments that require O₂ for generation of reactive oxygen species, the actual tumor cell killers. Liquid perfluorocarbons (PFCs) are inert gas solvents that help alleviate this oxygen deficit situation. PFC nanoemulsions have demonstrated oxygen delivery to tissues. The lifetime of ¹O₂ in PFCs is considerably expanded. PFC nanodroplets extravasate and accumulate in tumors. Alternatively, PFCs stabilize injectable O₂ microbubbles. On-demand local O₂ delivery is facilitated by ultrasound. Liquid PFC nanodroplets that convert into microbubbles upon activation provide another shuttle for O₂-delivery. PFC nanocarriers can be enriched with fluorescent dyes, radiopaque materials, photo(sono)sensitizers, loaded with chemotherapeutics, and fitted with targeting devices, or stimuli-responsive functions for image-guided theranostics. We review recent literature on PFC-based O₂ carriers to enhance the efficacy of radio-, photo(sono)dynamic- and chemo- therapies. PFC-based carriers may provide novel strategies to promote T-cell trafficking into tumors to improve immune responses.

Introduction: Perfluorocarbons as O₂ carriers

Hypoxia in malignant tumor tissue results from the imbalance between O₂ supply and consumption consequent to fast-growing tumors. Hypoxia is a characteristic of most solid tumors, and contributes directly to the development of malignancy.[1] Tumors can adapt their metabolism to their O₂-depleted microenvironment through activation of hypoxia inducible factors (HIFs) that are key in shifting to anaerobic energy production processes.[2]. The hypoxic microenvironment, which functions as a regulator of tumor survival and growth,[3-5] adversely affects the efficacy of essentially all current major cancer treatments, either directly, as for radiotherapy and photo- or sonodynamic therapy, or indirectly, as for chemotherapy and immunotherapy.[4,6] Recent reports have reviewed the effect of O₂ carriers, including red blood cells (RBC), hemoglobin-based O₂ carriers, or metal organic frameworks in alleviating hypoxia to improve the outcomes of cancer therapies.[7,8]

Here, we focus on perfluorocarbons (PFCs) that, either in the form of nanoemulsion droplets (NEs), microbubbles (MBs) and other PFC-based nanocarriers, can benefit to cancer therapies by delivering O₂. PFCs are investigated for numerous biomedical uses that generally rely on a combination of specific properties, such as high biological acceptance, remarkable ability to solubilize gases, extremely low solubility in water, low surface tension, or heat transfer capacity, that cannot be attained with their hydrocarbon counterparts.[9-11] Owing to very weak van der Waals intermolecular interactions, liquid PFCs dissolve large amounts of O₂, which are delivered through passive diffusion to hypoxic tissues. Gas dissolution in PFCs follows Henry's law (*i.e.* is directly proportional to the gas partial pressure), and does not depend on directional chemical binding as in hemoglobin. Consequently, O₂ is rapidly and extensively extracted from PFCs by hypoxic tissues.[9,12] A perfluorooctylbromide (*F*-octylbromide, the conventional italicized *F*- suffix will thereafter

mean perfluoro) nanoemulsion prevented hypoxia in pancreatic rat Langerhans islets; viability and insulin production capacity were preserved and extracellular matrix disruption was avoided.[13,14] A Phase III efficacy clinical trial of an *F*-octylbromide NE (*Oxygent* AF0144, Alliance Pharm. Corp., San Diego, USA) in general surgery patients established that the emulsion significantly reduced the need for allogeneic blood transfusion.[12,15] Although PFC NEs have been tested as adjuncts to radiotherapy with encouraging results, clinical trials remained limited.[16,17] An *Oxygent* formulation administered, along with carbogen breathing, to tumor-bearing mice immediately after chemo-/radiotherapy, significantly decreased the volume of hypoxic region.[16] The medical uses of low-dose PFC NEs was progressively extended to blood pool contrast for computed tomography, magnetic resonance imaging (MRI), Doppler ultrasound (US), molecular imaging and targeted drug delivery, as well as to *in vivo* MRI-guided immune cell tracking.[18,19]

PFCs are also widely employed for stabilizing gas microbubbles (MBs) used in the clinic as contrast agent for US imaging. MBs can be produced and administered as such, or can result from *in vivo* activation of PFC NEs (the so-called phase-shift emulsions, P-SNEs) that converts liquid droplets into MBs using US or light.[20] Administration of PFC-stabilized MBs in conjunction with US was deemed beneficial in several theranostic approaches that can combine diagnostic imaging and ablation, histotripsy, embolotherapy, radiotherapy, photo(sono)dynamic therapy, chemotherapy, gene delivery, including across the blood brain barrier, or anti-vascular therapy.[21-25] This short review, after briefly reminding the impeding effects of hypoxia on cancer therapies, reports recent advances that demonstrate the capacity for PFC-based NEs, P-SNEs, MBs and other PFC-based nanodevices to deliver oxygen to tumors and, thereby, efficiently fight hypoxia in O₂-dependant cancer therapies.

Hypoxia strongly hampers the efficacy of major cancer therapies

Radiotherapy (RT) uses ionizing radiations to produce free radicals, either directly in DNA, or indirectly in other cellular components, primarily water, to induce DNA damage and kill cancer cells.[26,27] Furthermore, O_2 can stabilize the DNA damage.[26,27] Hypoxia interferes with these processes and is a major cause of resistance to RT. An adequate O_2 supply is indeed critical for RT efficacy.

Photodynamic therapy (PDT) relies on light-induced activation of a photosensitizer (PS) that transfers energy to O_2 molecules, for generation of reactive oxygen species (ROS) such as singlet oxygen (1O_2), and superoxide ($O_2^{\bullet -}$) and hydroxyl (HO^{\bullet}) radicals.[28] The ROS interact with cell membrane lipids and proteins, and with DNA, exerting a cytotoxic effect. The deficit in O_2 in hypoxic tumors critically hampers PDT by limiting critical ROS generation. Sonodynamic therapy (SDT) is an emerging therapeutic modality similar to PDT that uses sound instead of light for activation.[29] Its main advantage is deeper penetration of sound in soft tissues thus reaching buried tumors. Again, SDT efficacy is limited in hypoxic condition.

Hypoxia-induced chemoresistance is also a major challenge to chemotherapy. It has been associated with hypoxia-inducible factor 1 (HIF 1), drug efflux, reduced apoptosis in hypoxic tumor, hypoxia-induced autophagy, lowering of DNA repair enzymes activity, and remoteness of cancer cells from blood vessels that hampers drug delivery.[30,31] Since the activity of many chemotherapeutics depends on ROS, increasing tumor oxygenation can effectively reduce hypoxia-induced chemoresistance.

Immunotherapy aims at activating the patient's own immune system to recognize and destroy cancer cells. Current cancer immunotherapies, including checkpoint inhibitors, dendritic cell vaccination, adoptive T cell therapy, and immunostimulatory agents are

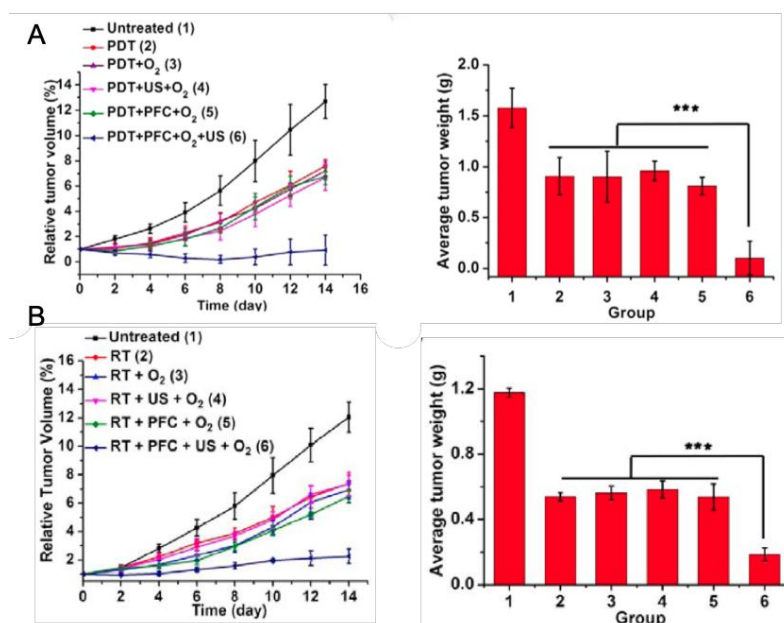
presently receiving considerable attention.[32] Preclinical and clinical evidence indicate that hypoxia confers an immunosuppressive character to the tumor microenvironment, which limits infiltration of T cells in the hypoxic regions.[33,34] In addition, the hypoxic regions were found to be infiltrated by immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and T-regulatory (Treg) cells, which protect tumor cells from natural killer cells and T cells during tumor growth and after chemo- or immunotherapy.[33,34] Hypoxia also promotes evasion of immune checkpoint proteins such as PD-L1 through HIF-1 α -dependent upregulation.[34] Adenosine, an important immunomodulatory metabolite that accumulates in hypoxic tumors, is a potent T cell inhibitor.[35,36] Adenosine is excreted by tumor cells into the extracellular tumor matrix, resulting in infiltration and accumulation of Treg.[36,37] Importantly, it was demonstrated that supplemental O₂ can act as a novel immune checkpoint inhibitor by disrupting the hypoxia-adenosine-A2aR pathway.[38]

PFC nanoemulsions for oxygen delivery to tumors

PDT was augmented by a *F*-hexane NE coated with a phospholipid shell loaded with a near-infrared (NIR) photosensitizer (PS; IR780) in a mice tumor model.[39] This enhanced activity was assigned to supplemental O₂ provided by the PFC for generating ¹O₂, and to increased half-life of ¹O₂ in the PFC, versus cellular environments. An IR780-loaded, *F*-tripropylamine/human serum albumin (HAS) NE enhanced PDT efficiency by prolonging ¹O₂ generation and by allowing uniform dispersion of the dye in the albumin layer, thus preventing self-quenching and extending the PS triplet-state lifetime.[40] Solubilization of a porphyrin grafted with fluorinated chains (*F*-chains) inside a PFC (*F*-decalin/*F*-tripropylamine) NE reduced PS leakage, efficiently boosted ¹O₂ generation and increased PDT efficiency *in cellulo*.[41]

A low-power/low-frequency US pulse released at the tumor site the O₂ adsorbed in the lungs by a *F*-15-crown-5-ether/HAS NE.[42] This O₂ shuttle dramatically enhanced tumor oxygenation and improved the outcomes of PDT and RT of various types of solid tumors in mice (Fig. 1).

Figure 1.



US-triggered tumor oxygenation with PFC nanoemulsions.

Photodynamic therapy (A) and radiotherapy (B). Tumor growth after various treatments in mice (left); and corresponding average tumor weights (right). From[42].

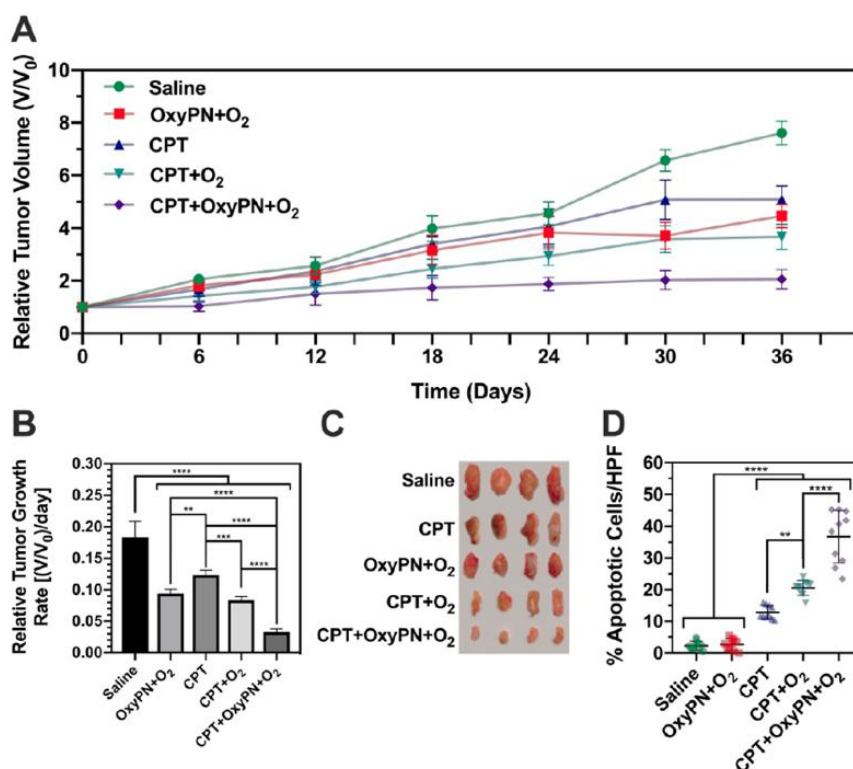
Extended blood circulation is key for effective tumor accumulation of O₂- and drug-carrying PFC NEs. Biomimetic functionalization of nanocarriers can increase blood compatibility, reduce immune clearance, and prolong circulation.[43] Indocyanine green (ICG)-containing, HSA-shelled *F*-tributylamine NE droplets additionally coated with RBC membrane fragments exhibited increased blood circulation half-life, tumor accumulation in mice, resulting in enhanced PDT.[44] ICG irradiation also produced a photothermal effect, which, combined to PDT, led to 93% tumor growth inhibition.[44] Likewise, coating *F*-15-crown-5-ether NE droplets stabilized by poly(D,L-lactide-co-glycolide) with RBC membrane

fragments prolonged blood circulation time.[45] The RBC-coated NE strongly decreased HIF1- α expression, and significantly increased tumor-growth inhibition versus RT alone when injected in mice breast cancer.

Damage to surrounding healthy tissues is a major downside of RT. Inorganic nanoparticles (NPs) containing high-atomic-number elements with strong X-ray attenuation can help concentrate the radiation energy at the tumor site.[46] Pegylated *F*-hexane NEs decorated with hydrophobically-modified TaOx NPs allowed higher radiation energy delivery at the tumor site with lesser side effects,[47] and delivered more O₂ than *F*-hexane-loaded Bi₂Se₃ NPs.[48] NEs of a PFC mixture (mostly *F*-tributylamine) shelled with HAS enhanced RT efficacy in mice-bearing breast or colon tumors through increased RBC infiltration in the tumors, which was deemed to provide second-stage O₂ delivery.[49] An immunotherapy-related study demonstrated that the same PFC NE could promote intratumoral infiltration of CD8⁺ and CD4⁺ T cells via platelet inhibition.[50] Importantly, a tumor inhibition rate reaching 90% was obtained by combining the administration of the PFC NE with that of an anti-PD-L1 antibody, indicating that PFC NEs have potential for enhancing anti-PD-L1 immunotherapy.

Hypoxia-induced resistance to cisplatin was alleviated by administration of a preoxygenated phospholipid-coated *F*-octylbromide NE along with carbogen breathing (Fig. 2).[51]

Figure 2



Effect of PFC nanoemulsions on hypoxia-induced chemoresistance.

A) Lung tumor growth in mice treated with preoxygenated *F*-octylbromide nanoemulsion (OxyPN+O₂), free cisplatin (CPT), free cisplatin under hyperoxic breathing (CPT+O₂) or cisplatin + preoxygenated *F*-octylbromide nanoemulsion (CPT+OxyPN+O₂); B) tumor growth rate; C) tumors removed in animals of each group; and D) percentage of apoptotic cells in tumors. From[51].

PFC phase-shift nanoemulsions: Exploiting the beneficial features of both liquid nanodroplets and gas bubbles

Small-size PFC NEs benefit from enhanced permeability and retention effect (EPR)-mediated tumor accumulation due to leaky tumor vessels, and from US-induced permeabilization of the tissue structure. MBs present unique responsiveness to US for contrast imaging and O₂-release control. Vaporization of NE droplets of a volatile PFC

generates MBs that can provide tumor oxygenation for enhanced cancer therapy.[20] Small doses of a *F*-pentane P-SNE (NVX-108, NuvOX Pharma, Tucson), emulsified with a pegylated *F*-surfactant, increased the tumor O₂ partial pressure by up to 400% (when combined with carbogen and radiation) in a hypoxic pancreatic tumor xenograft in mice, resulting in a two-fold reduction in average tumor volume.[52] A Phase Ib/II chemoradiation clinical trial of NVX-108 in 11 patients with glioblastoma indicated safety and significant decrease in tumor volume.[53]

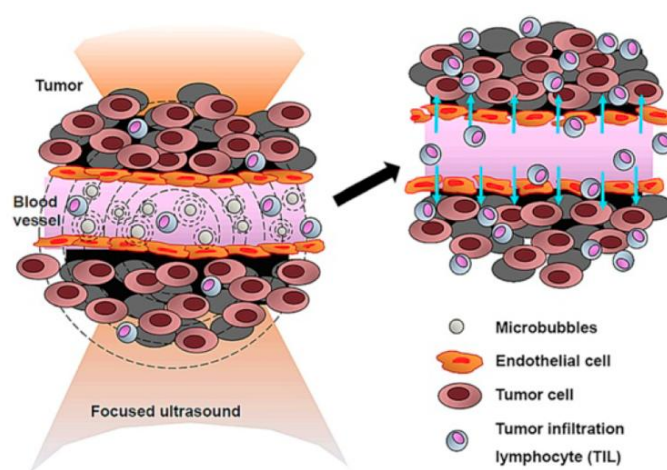
In addition to the EPR effect that enables passive therapeutic nanocarrier uptake into tumors, and targeting to malignant cells via conjugation with antibodies or cell-specific peptides, an alternative strategy for nanocarrier delivery to tumors involves their uptake by immunocompetent cells (*e.g.* monocytes/macrophages). These cells are then recruited into the tumor to act as cellular “Trojan horses” to deliver their therapeutic payload.[54] *F*-pentane P-SNEs and doxorubicin-containing poly(acrylic acid-*co*-distearin acrylate) NPs were thus co-loaded into bone marrow-derived monocytes by phagocytosis.[55] After administration to pre-irradiated tumor-bearing mice and exposure to remote-controlled focused ultrasound (FUS), the therapeutic monocytes accumulated in the tumor and induced apoptosis of cancer cells.

A further option: administration of PFC-stabilized O₂ microbubbles

The FDA-approved PFC-stabilized MB products used for contrast-enhanced US imaging are currently investigated for multiple indications, including O₂-delivery in cancer-related applications. For example, US-triggered destruction of O₂-loaded lipid-shelled *F*-butane MBs provided Rose Bengal-mediated SDT along with paclitaxel and doxorubicin chemotherapy, leading to reduced cancer cell viability in a spheroid model of human breast cancer and to decreased tumor growth in mice.[56] These MBs were also investigated for antimetabolite

therapy (with antimetabolite 5-fluorouracil attached to the MB surface).[57] US-triggered destruction of O₂ MBs osmotically stabilized by a tiny amount of *F*-butane significantly enhanced O₂ delivery for local tumor hypoxia alleviation, thus providing image-guided O₂ delivery.[22] *F*-propane-stabilized lipid-shelled O₂ MBs substantially elevated pO₂ in breast tumor upon US activation in rabbits.[58] Phospholipid-coated MBs of sulfur hexafluoride (SF₆, another *F*-chemical used for MB stabilization) (SonoVue, Bracco Diagnostics, Milan, Italy) elicited an immunological anticancer response when exposed to low-pressure pulsed FUS.[59] Increased tumor microvasculature permeability and suppressed tumor progression in colorectal tumor-bearing mice were demonstrated (Fig. 3). A continuous infiltration of cytotoxic CD8⁺ T lymphocytes was observed, which was deemed to result from the alterations in the tumor microenvironment produced by the SF₆ MBs. The CD8⁺/Treg ratio increased significantly, and tumor growth was inhibited, especially in the initial days following treatment.

Figure 3



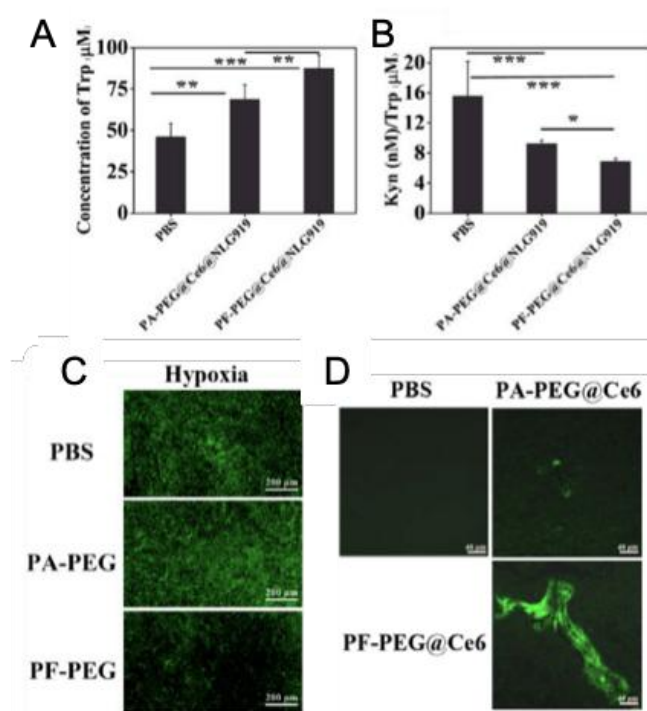
Immunological response triggered by SF₆ microbubbles.

Exposure of tumor tissue to focused ultrasound in the presence of SF₆ microbubbles enhances blood vessel permeability and recruitment and penetration of tumor-infiltrating lymphocytes. From [59].

More PFC-based nanocarrier opportunities

The O₂-delivering efficacy of self-assembled nanocarriers such as micelles made of fluorinated amphiphilic co-polymers has been reported. Micelles of a copolymer comprising *F*-phenyl groups, a porphyrin and poly(ethylene glycol) (PEG) chains increased PDT efficacy; the production of singlet oxygen increased with *F*-phenyl to porphyrin ratio.[60] Chlorin e6 (Ce6)-loaded micelles of a *F*-alkylated polyethyleneimine-derived polymer provided effective PDT.[61] Micelles of a pegylated *F*-poly(β -amino ester) were co-loaded with Ce6 and NLG919, an inhibitor of indoleamine 2,3-dioxygenase (IDO, an oxidoreductase expressed in various neoplastic cells that catalyzes the catabolism of tryptophan into kynurenine). These fluorinated micelles inhibited the growth of both primary and metastatic tumors in mice (Fig. 4).[62]

Figure 4



Hypoxia-relieving capacity of PFC-based micelles.

A) Tryptophan (Trp) concentration in plasma samples; B) ratio of kynurenine (Kyn) to Trp; C) immunofluorescence staining of tumor sections showing hypoxia (green) and D) singlet

oxygen production (green) in phosphate buffer (PBS), non-fluorinated (PA-PEG) and fluorinated (PF-PEG) micelles groups. From [62].

Photosensitive doxorubicin-loaded micelles with PEG chains outside and *F*-chains inside were reported to enhance O₂ delivery, resulting in synergistic PDT and chemotherapy in mice.[63] *F*-alkylated chain-functionalized hollow mesoporous organosilica NPs provided an O₂ reservoir and carrier system for sonosensitizer IR780.[64] US treatment increased pO₂ in pancreatic tumor. Reversal of tumor hypoxia by accelerated O₂ supply increased SDT efficacy, inhibiting pancreatic tumor growth in mice.[64] *F*-pentane-filled hollow mesoporous organosilica NPs, surface-decorated with ultra-small CuS NPs (for photoacoustic imaging) and a radioisotope (⁶⁴Cu, for positron emission tomography, PET) were investigated.[65] Mild hyperthermia generated by low-power NIR laser activation of the CuS NPs vaporized the PFC, stimulating O₂ diffusion. The PFC-containing NPs reduced hypoxia and enhanced RT in xenograft tumor bearing-mice, offering theranostic perspectives for simultaneous PET/photoacoustic/ultrasound imaging. Tumor-targeted porous hollow magnetic Fe₃O₄ NPs loaded with *F*-hexane and etoposide (EP), a topoisomerase II inhibitor used in several cancer indications, delivered O₂ and effectively reduced the hypoxia-induced EP resistance.[66]

Conclusions and perspectives – Highly versatile theranostic approaches

An abundant literature has established that injectable perfluorocarbon-based nanocarriers, including O₂-loaded or stabilized nanoemulsions, microbubbles, phase-shift nanoemulsions, and other PFC-based nanodevices have the potential for safe and effective, on-demand triggerable focused delivery of the O₂ that is critical for O₂-dependant cancer therapies, including immunotherapy. Use of PFC-based nanocarriers for boosting immune cell infiltration in tumors has been demonstrated. The additional capacity of these versatile

devices for simultaneous multimodal imaging and guidance opens a wide range of theranostic perspectives. Practical implementation of clinically translatable PFC O₂ carriers will require rational design, careful selection of the critical components, and establishment of thorough manufacturing processes. It also entails optimized control of the multiple parameters that govern their size, size distribution, stability, vaporization process in the case of phase-shift nanoemulsions, tumor-targeting effectiveness, response to ultrasound, laser light and other stimuli, and further understanding of their interactions with immune cells. Incorporation of PFCs in nanodevices to allow *in situ* O₂ generation in the tumor microenvironment through strategies such as catalytic decomposition of endogenous hydrogen peroxide or light-triggered water splitting, could also be considered.

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